Original Article
The CDK6-c-Jun-Sp1-MMP-2 axis as a biomarker and therapeutic target for triple-negative breast cancer

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Abstract: Triple-negative breast cancer (TNBC) has high metastatic, drug-resistance, and recurrence rates, and is characterized by an angiogenic and fibrotic microenvironment that favors cancer malignancy. However, details of the mechanisms underlying malignancy are still largely unknown. Our mouse model indicated that knockdown of CDK6 inhibited lung metastasis significantly compared to parental cells. Immunohistochemical analyses revealed that the levels of collagen and the angiogenic marker matrix metalloproteinase (MMP)-2 were much lower in CDK6-deficient cells. To examine mechanisms in the CDK6-mediated phenotype of cancer cells, we studied its role in MMP-2 expression. CDK6 mediated the recruitment of transcription factors including c-Jun and Sp1 to the *MMP2* promoter. Knockdown of CDK6 significantly suppressed the expression of *MMP2* mRNA. Consistent with the in vitro data, the expression of CDK6 was positively correlated with the angiogenic and fibrotic tumor microenvironment in TNBC patient tissues as shown by MMP-2 and fibronectin staining, respectively. More importantly, after screening a small molecule library of 31 protein kinase inhibitors, we found that the Raf inhibitor sorafenib displayed the highest cytotoxicity in CDK6-depleted cells. These data indicate that CDK6 serves as an anti-microenvironment target and affects the drug response in retinoblastoma-proficient TNBC, suggesting that combining a CDK6 inhibitor and sorafenib leads to a synthetic effect that may represent a personalized therapeutic approach for patients with TNBC.

Keywords: CDK6, MMP2, c-Jun, tumor microenvironment, sorafenib

Introduction

Breast cancer is one of the most prevalent cancers in Taiwan with a rising incidence [1]. Its heterogeneity makes providing effective medical treatment difficult. Currently, the type of breast cancer and corresponding intervention strategies are determined using surface markers on cancer cells, including estrogen receptors, progesterone receptors, and human epidermal growth factor receptor 2. However, triple-negative breast cancer (TNBC), which lacks all three receptors, is the most aggressive and difficult subtype to treat because of its heterogeneity and the absence of any specific biomarker for targeted therapy [2]. Therefore, the

development of specific and personalized treatments for TNBC is one of the most important issues in oncology research.

Cyclin-dependent kinases (CDKs) participate in controlling the cell cycle. CDK6 and CDK4 were the first of this family to be identified and were found to be activated upon mitogenic stimulation. After binding with cyclin D, CDK4/6 negatively regulates the retinoblastoma protein (Rb) and consequently releases the transcription factor E2F, promoting exit from the G1 to S phase [3-5]. A dramatic increase in CDK6 expression has been observed in several cancer types, including leukemia, glioblastoma, pancreatic cancer, and breast cancer [6-9]. How-

ever, breast cancer is a heterogeneous disease. Therefore, understanding the function of CDK6 in different breast cancer subtypes could lead to more potential strategies in personalized therapy.

Three selective CDK4/6 inhibitors have been approved by the United States Food and Drug Administration for treating estrogen receptor-positive breast cancer: palbociclib (PD033-2991), ribociclib (LEE011), and abemaciclib (LY835219) [9-12]. Because these inhibitors achieve their antitumor effects by increasing Rb protein function, the expression of functional Rb has been proposed as a basis for treatment with them [13]. Notably, upregulation of cyclin D and CDK4/6 has been observed in TNBC. However, 20% of TNBC cases exhibit loss or mutation of Rb, which hinders the use of CDK4/6 inhibitors for treatment [14]. Nevertheless, available data suggest that high CDK4/6 and Rb-proficient TNBC may be still treatable with CDK4/6 inhibitors.

Combining CDK4/6 inhibitors with anti-estrogen therapies is beneficial in the treatment of estrogen receptor-positive breast cancer [15]. Other evidence indicates that the antitumor activity of trastuzumab against HER2-positive breast cancer can be enhanced when combined with CDK4/6 inhibitors [16, 17]. Therefore, combinations of CDK4/6 inhibitors with other targeted therapies may improve efficacy and reduce drug resistance in TNBC [18]. In the current study, we revealed the interplay of CDK6 with its microenvironment in TNBC tissue and evaluated the therapeutic effect of CDK6 inhibition for TNBC. We also screened clinical and investigational drugs for candidates capable of producing therapeutic effects synergistically with CDK6 inhibition.

Materials and methods

Cell culture

The TNBC cell lines used in this study were 4T1, MDA-MB-231 and MDA-MB-468 (American Type Culture Collection (ATCC), Manassas, VA, USA). In addition, M10 mammary epithelial cells (Biosource Collection and Research Center, Hsinchu, Taiwan), SVEC4-10 lymphatic endothelial cells, and RMF-EG fibroblast cells (ATCC) were used. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM),

DMEM/F12 medium, or M199 medium (Thermo Fisher Scientific, Waltham, MA, USA) with 10% fetal bovine serum (Hyclone Laboratories, South Logan, UT, USA) and antibiotics at 37°C, equilibrated with 95% humidified air with 5% CO_2.

Specimens

Tissue blocks from 37 patients with TNBC were selected from the Department of Pathology, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan. Institutional Review Board approval for using these tissues in this study was given by the Research Ethics Committee of the Kaohsiung Medical University Hospital (IRB:KMUHIRB-E(I)-20180321). Data were analyzed anonymously; therefore no additional informed consent was required. All methods were performed in accordance with the guidelines and regulations of the Kaohsiung Medical University Hospital.

Reagents

Antibodies against CDK6 (GTX103992), matrix metalloproteinase (MMP)-2 (GTX104577), MMP-9 (GTX100458), and fibronectin (GTX-112794) were from GeneTex International (Irvine, CA, USA). β-Actin (#3700), Sp1 (#9389), c-Jun (#9165), antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Proliferating cell nuclear antigen (PCNA) (#ab-29), lymphatic vessel endothelial hyaluronan receptor (LYVE-1) (#ab14917), and CD31 (#ab28364) antibodies were from Abcam (Cambridge, MA, USA). T-5224 was from Selleckchem (Houston, Texas, USA). Mithramycin A was purchased from Sigma-Aldrich (St. Louis, USA).

Western blotting

Protein extraction and immunoblotting were performed as described previously [19]. In brief, cells were lysed in protein lysis buffer containing a protease and phosphatase inhibitor cocktail (M-PERTM mammalian protein extraction buffer; Thermo Scientific, Rockford, IL, USA) on ice for 20 min and cellular debris was removed by centrifugation. Proteins (20-30 ug) were quantified, separated by 8-12% sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and immunoblotted using the indicated anti-

bodies for overnight, followed by incubation with horsedish peroxide (HRP)-conjugated secondary for 1 h at room temperature.

Gene knockdown

To knock down the expression of CDK6 in MDA-MB-231 and 4T-1 cells, short hairpin (sh)RNA against human CDK6 (sh-CDK6) (sequence: #1: CAGACAGAGAAACCAAACTAA; #2: CAGACAGA-GAAACCAAACTAA, and #3: CGTGGAAGTTCAG-ATGTTGAT), shRNA against luciferase (sequence: GCGGTTGCCAAGAGGTTCCAT), control mouse CDK6 shRNA (#1: CGGTTGCATC TTT-GCAGAAAT; #2: CTTGACCACTTACTTGGATAA; #3: GCTCAACCCATCG AGAAGTTT), and control Luciferase shRNA (sequence: CTTCGAAATG-TCCGTTCGGTT) were purchased from the National RNAi Core Facility (Academia Sinica, Taipei City, Taiwan). The transfection protocols for CDK6 and luciferase followed the manufacture's guidelines.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA extraction and qRT-PCR protocols were performed as described previously [19]. Briefly, total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse-transcribed using SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's protocol. Synthesized cDNA was used as a template for PCR amplification with primers for human *CDK6*, hepatocyte growth factor (*HGF*), fibroblast-activating factor (*FAF*), interleukins (*IL6* and *IL8*), transforming growth factor-β (*TGFB1*), platelet-derived growth factor (*PDGF*), vascular endothelial growth factor-A (*VEGFA*), *MMP2*, and *MMP9*. Quantitative RT-PCR was performed in a 20 μL reaction volume using the standard protocols provided with the LightCycler 480 II system (Roche, Basel, Switzerland). Expression of the abovementioned genes was determined as: $\Delta CT = CT$ (target gene)-CT (GAPDH); $\Delta\Delta CT = \Delta CT$ (experimental group)-ΔCT (control group). The primers of CDK6 were (forward) 5'-CGTGGTC-AGGTTGTTTGATG-3' and (reverse) 5'-CGGGCT-CTG GAACTTTATCC-3'; HGF were (forward) 5'-GAGGGACATAAGAAAAGAAGA-3' and (reverse) 5'-GTGTGGTATCATGGAACTCCA-3'; IL-6 were (forward) 5'-GACAGCCACTCACCTCTTCA-3' and (reverse) 5'-TGCAGGAAC TGGATCAGGAC-3'; IL-8 were (forward) 5'-CAGAGACAGCAGAGCACAC-3'

and (reverse) 5'-AGTTCTTTAGCACTCCTTGGC-3'; MMP-2 were (forward) 5'-TCTCCTGACATTGA-CCTTGGC-3' and (reverse) 5'-CAAGGTGCTGG CTGAGTAGATC-3'; MMP-9 were (forward) 5'-TTGACAGCGACAAGAAGTGG-3' and (reverse) 5'-GCCATTCACGTCGTCCTTAT-3'; PDGF were (forward) 5'-CAGCAGGCGTTGGAGATCAT-3' and (reverse) 5'-GGAGTCGGCATG AATCGCTG-3'; TGF-β1 were (forward) 5'-GTCAATGTACAGCTGCC-GCA-3' and (reverse) 5'-CCCAGCATCTGCAAAG-CTC-3'; VEGF-A were (forward) 5'-ACAGGTA-CAGGGATGAGGACAC-3' and (reverse) 5'-AA-GCAGGTGAG AGTAAGCGAAG-3'; VEGF-C were (forward) 5'-GCCAACCTCAACTCAAG GAC-3' and (reverse) 5'-CCCACATCTGTAGACGGACA-3'; ANG-2 were (forward) 5'-TGGGATTTGGTAACC-CTTCA-3' and (reverse) 5'-CCTT GAGCGAATA-GCCTGAG-3'; CXCL-5 were (forward) 5'-GCA-AGGAGTTC ATCCCAAAA-3' and (reverse) 5'-AAACTTTTCCATGCGTGCTC-3'; CXCL-6 were (forward) 5'-AGAGCTGCGTTGCACTTGTT-3' and (reverse) 5'-GC AGTTTACCAATCGTTTTGGGG-3'; FAF were (forward) 5'-GAATGTT TCGGTCCTG-TC-3' and (reverse) 5'-CCATCCAGTTCTGCTTTC-3'; MMP2 promoter chip sequence for c-Jun binding were (forward) 5'-CTTCTCAAACTGTTC-CCTGC-3' and (reverse) 5'-GGAACGCCTGACTT-CAGC-3'; MMP2 promoter chip sequence for Sp1 binding were (forward) 5'-GTCCTGGCAAT-CCCTTTGTA-3' and (reverse) 5'-GGGGAAAAG-AGGT GGAGAAA-3'.

Transwell co-culture wound healing assay

RMF-EG or SVEC4-10 cells were cultured in 6-well plates. A wound area was created using a 200 μL pipette tip on the confluent monolayer. The cells were then co-cultured with parental/sh-CDK6 MDA-MB-231 cells through a Transwell with 0.2 μm inserts on top. After co-culturing, images were captured at 0, 4, and 8 h. The experiments were performed in triplicate.

Tube formation assay

Matrigel Basement Membrane Matrix (BD Biosciences, Franklin Lakes, NJ, USA) was diluted with M199/DMEM and used to coat 24-well plates at 37°C for 1 h. Then, 5×10^4 SVEC4-10 cells were seeded onto the Matrigel. Conditioned medium from parental or sh-CDK6 MDA-MB-231 cells was added into the Transwells and incubated in the same well with SVEC4-10 cells. The tube-forming ability of SVEC4-10 cells

was measured at 0.5, 1, and 2 h with or without parental or sh-CDK6 MDA-MB-231 conditioned medium.

Mice

Five-week-old female BALB/c mice (22-25 g) were purchased from the National Laboratory Animal Center (Taipei City, Taiwan). Mice were housed in the Kaohsiung Medical University Animal Research Facility. All experimental methods and procedures were approved by the Center for Laboratory Animals Committee at Kaohsiung Medical University (No. 107097) and performed according to the care and use of laboratory animal guidelines. Animals were kept in an Association for the Assessment and Accreditation of Laboratory Animal Care International-approved animal facility in Kaohsiung Medical University. Mice were divided randomly into two groups and subjected to treatment. Control and CDK6 knockdown 4T-1 cells (5 × 10^4 cells/mouse) were injected into the left fourth thoracic mammary fat pad. For ribociclib treatment, mice were then randomized into 2 groups: control and ribociclib (50 mg/kg/day) was given once daily for 5 days a week via oral gavage for 2 weeks. Tumor volumes were measured every 3 days from the second week after injection and were calculated using the formula: V = (length) × (width)^2 × 0.5. After 45 days, animals were sacrificed and the tumors were excised and used for immunohistochemistry (IHC). For individual inhibitors, mice were randomized into two groups containing four mice each and treated with 0.2 mg/kg/day of Mithramycin A (i.p.) and 3 mg/kg/day of T-5224 (Oral gavage) five times per week for 42 days. After 42 days, were sacrificed and the tumors were excised and used for immunohistochemistry (IHC).

Immunohistochemistry (IHC) staining

IHC was performed as described previously [20]. In brief, paraffin-embedded tissue samples were cut into 4-μm-thick sections, de-paraffinized, and rehydrated. Antigen retrieval was achieved by autoclaving the sections at 121°C for 10 min in a pH 6.0 retrieval solution (DAKO, Carpinteria, CA, USA). Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide (Sigma-Aldrich, St. Louis, MO, USA) for 10 min. The sections were then incubated with primary antibodies (CDK6, MMP-2, CD31, PCNA, and fibronectin) at room temperature for 1 h. The REAL™ EnVision™ Detection System (DAKO) was then applied for 1 h. Finally, the sections were incubated in 3', 3-diaminobenzidine for 5 min, counterstained with Mayer's hematoxylin, and mounted. Negative controls were prepared by replacing the primary antibodies with nonimmune serum. The trichrome stain kit was purchased from Abcam (#ab150686).

Scoring

CDK6, MMP-2, and fibronectin expression in patients was scored through signal intensity (0, 1+, 2+, and 3+) and proportions of positive cells (0 = ≤ 10%, 1 = 11-25%, 2 = 26-50%, 3 = > 50%) [20]. Staining indices were calculated as the product of the intensity of signal and the proportion of positive cells; scores ranged from 0 to 9. A score ≤ 4 indicated low expression of CDK6 and MMP-2; a score ≥ 6 indicated high expression of CDK6 and MMP-2. Fibronectin expression was scored 1 or 0 according to whether or not it was staining was observed in the extracellular matrix.

Statistical analyses

The expression of CDK6, fibronectin, and MMP-2 in TNBC tissues, as determined by IHC staining, was compared using the chi-square test. A p value < 0.05 was considered to indicate a statistically significant difference between groups. All statistical analyses were performed using SPSS 19.0 software (IBM, Chicago, IL, USA).

Results

Suppression of CDK6 affected tumor growth and lung metastasis in an animal model of TNBC

To determine whether CDK6 was a biomarker and therapeutic target of TNBC, we performed patient-based analyses of RNA using the UALCAN database [21]. Among breast cancer types, TNBC showed the highest level of *CDK6* at the RNA level (Figure S1). Notably, the level of CDK6 protein in Rb-proficient MDA-MB-231 cells was higher than in Rb null MDA-MB-468 cells (Figure S2). This finding suggests that targeting CDK6 may serve as an anticancer strategy in Rb-proficient TNBC.
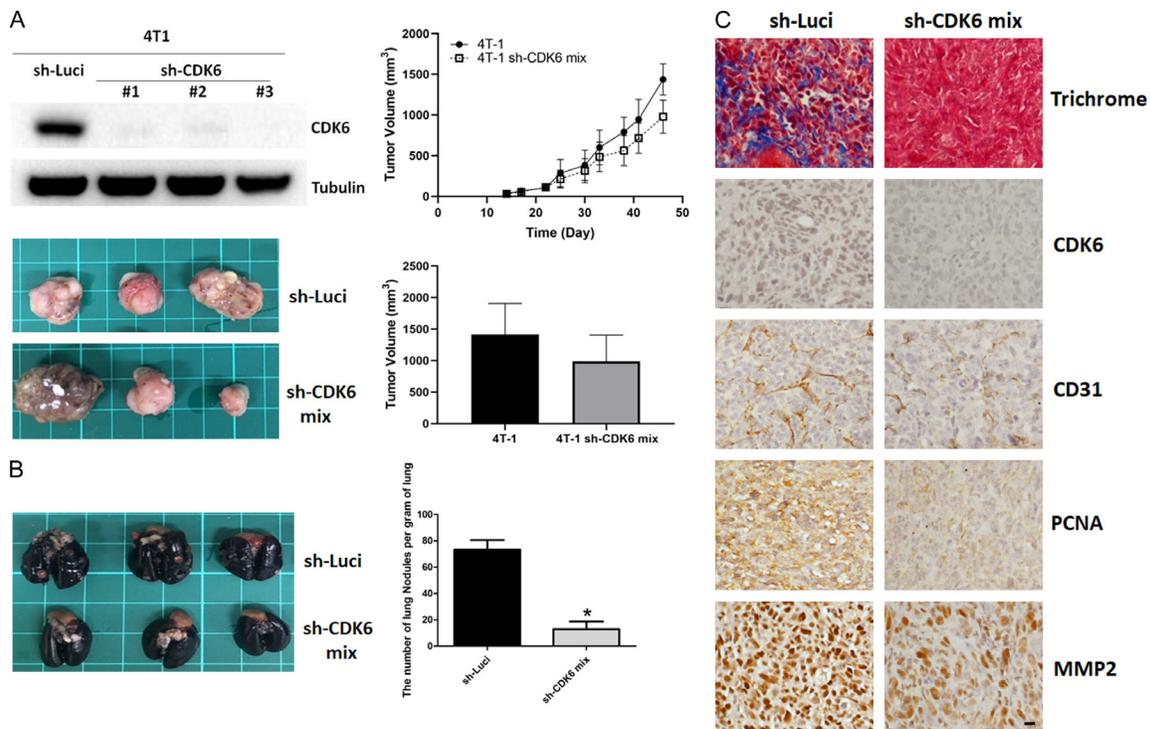
Figure 1. Knockdown of CDK6 reduces lung metastasis and malignant phenotype in vivo. A. Left panel, levels of CDK6 in parental and sh-CDK6 transfected 4T1 cells, and representative images of breast cancer tissue with high or low expression of CDK6 in BALB/c mice. Right panel, quantitative analysis of tumor growth and size 45 days after tumor cell injection. Comparisons utilized the 2-tailed Student t test (*$P < 0.05$). B. Lungs were perfused with India ink after the mice were euthanized. Left panel, representative images of lung nodules. Right panel, numbers of visualized nodules per gram of lung tissue compared using the 2-tailed Student t-test (*$P < 0.05$). C. Representative immunohistochemical staining of CDK6, PCNA, CD31 and MMP-2, and collagen (Masson's trichrome stain) in tumors of parental and CDK6-deficient breast cancer tissues. Original magnification: 40 ×, scale bar: 20 μm.

High metastasis and proliferation rates are characteristics of the TNBC phenotype. Murine 4T1 is a typical TNBC cell line that has been used as an allograft to induce the neoplastic process in immune-competent mice (e.g., BALB/c). To evaluate the effect of CDK6 on the growth and metastasis of TNBC cells in vivo, we established a murine 4T1 TNBC allogeneic model in BALB/c mice. Mock vector-and sh-CDK6-transfected cells were implanted orthotopically into the mammary glands of mice. The tumor growth of CDK6-depleted cells was not significantly changed relative to that of parental cells (**Figure 1A**). However, notably, we found that silencing CDK6 reduced lung metastasis significantly relative to control, mock-transfected tumor cells (**Figure 1B**). IHC revealed that levels of CD31 and MMP-2 were much lower in CDK6-deficient cells (**Figure 1C**). The level of PCNA was partially reduced in CDK6-depleted cancer tissue. More importantly, trichrome staining revealed decreased collagen in CDK6-

depleted tumors (**Figure 1C**). These data suggest that continuous expression of CDK6 was required for the maintenance of a malignant phenotype. Furthermore, CDK6 silencing suppressed lung metastasis and modified the tumor microenvironment in vivo.

Knockdown of CDK6 in cancer cells abolishes cancer-induced fibroblast activation and migration

TNBC is characterized by higher grades of fibrosis than other types of breast cancer. Fibrotic tissue not only blocks the delivery of drugs into the tumor, but also provides a pro-metastatic microenvironment [22]. We found that CDK6 expression was positively correlated with fibrosis in tumor tissue (**Figure 1C**); therefore, we studied possible mechanisms that affected the activation of fibroblasts regulated by CDK6. We examined the migration ability of fibroblast cells by co-culturing with parental and CDK6-

depleted breast cancer cells. As shown in **Figure 2A**, CDK6-deficient breast cancer cells did not increase the migration of fibroblasts, while parental breast cancer cells did (**Figure 2A**). Consistent with these findings, the migration of fibroblast was suppressed in CDK6-deficient 4T1 cells (Figure S3). On the other hand, cancer-associated fibroblasts did not promote the migration of CDK6-depleted cells (**Figures 2B** and S4).

Several proteins involved in fibroblast activation, including HGF, FAF, IL-6, IL-8, MMP-2, MMP-9, TGF-β, and PDGF, were examined. Among them, IL-6, MMP-2, MMP-9, TGF-β, and PDGF levels were significantly decreased in RMF-EG cells co-cultured with CDK6-deficient cells (**Figure 2C**). These results demonstrate that CDK6 plays an important role in the interplay between cancer cells and fibroblast activation.

Knockdown of CDK6 in cancer cells decreases cancer-induced tube formation and migration of endothelial cells

Lymphatic invasion is an aggressive biological phenotype. LYVE-1 is a specific marker for lymphatic blood vessels. As shown in **Figure 3A**, knockdown of CDK6 suppressed lymphangiogenesis. To validate the clinical significance of CDK6, we examined the correlation between CDK6 expression and lymphatic invasion by TNBC cells. Levels of CDK6 in lymph node tissue were higher than in primary tissue (**Figure 3B**). To address the mechanism by which CDK6 in TNBC cells induced lymphangiogenesis, lymphatic endothelial cells (SVEC4-10) were used. Co-culturing MDA-MB-231 cells with SVEC4-10 cells significantly increased the migration of SVEC4-10 cells. However, co-culturing SVEC4-10 cells with CDK6-knockdown MDA-MB-231 cells decreased their migration (**Figure 3C**).

These endothelial cells were also treated with conditioned medium from either control or CDK6-deficient cells, and their tube-forming abilities were then compared. We found that knockdown of CDK6 in MDA-MD-231 and 4T1 reduced cancer-induced tube formation (**Figure 3D**). Finally, we measured levels of lymphangiogenesis-associated factors including VEGF-A, VEGF-C, angiopoietin-2, CXCL5, CXCL6, IL-6, and IL-8. Only the protein levels of CXCL5 were reduced in CDK6-knockdown cells (**Figure 3E**).

Taken together, these data indicate that endothelial cells with high CDK6 expression may be responsible for the pro-angiogenic abilities of cancer, while downregulating CDK6 levels in cancer cells can abolish this effect.

CDK6 upregulates MMP-2 transcription

CDK6 is involved in kinase-dependent pathways that control cell-cycle progression. However, CDK6 is also a chromatin-bound cofactor that can directly control gene expression [23]. As CDK6 may be an important modulator of tumor-secreted factors that support angiogenesis and fibrosis, we screened for proangiogenic and profibrotic factors that may be regulated by CDK6 in cancer cells. MMPs not only play key roles in activating fibroblasts, but also promote tumor growth, metastasis, and angiogenesis [24, 25]. Our data indicated that knockdown of CDK6 significantly reduced the mRNA expression levels of *MMP2* and *MMP9* (**Figure 4A**). Consistent with the mRNA levels, MMP-2 and MMP-9 protein levels also decreased in CDK6-knockdown cells (**Figures 4B** and S5).

Sp1 and c-Jun are well-known regulators of MMP-2 promoter activity. As shown in **Figure 4B**, protein levels of c-Jun and Sp1 were decreased in CDK6-depleted cells. Notably, nuclear translocation of c-Jun and Sp1 was suppressed in CDK6-knockdown cells (**Figures 4C** and S6). Finally, the levels of c-Jun and Sp1 at the MMP-2 promoter were decreased in CDK6-deficient cells (**Figure 4D**). Consistent with these findings, IHC revealed that levels of c-Jun and Sp1 were much lower in CDK6-deficient cells and ribociclib treatment cells (**Figure 4E** and **4F**). A gene-selective Sp1 inhibitor, Mithramycin A and c-Jun binding inhibitor, T-5224 also decreased the expression of MMP2 and tumor growth in vivo (**Figure 5**). Taken together, these results suggest that inhibiting CDK6 may have a therapeutic effect by preventing formation of a progressive tumor microenvironment through modulation of the c-Jun/Sp1-MMP-2 axis.

CDK6 is overexpressed in TNBC and positively correlated with angiogenesis and fibrosis within the tumor microenvironment

Angiogenesis and fibrosis in the tumor microenvironment are indicators of cancer progression and poor prognosis [26, 27]. To improve our understanding of the role of CDK6 in tumor tis-
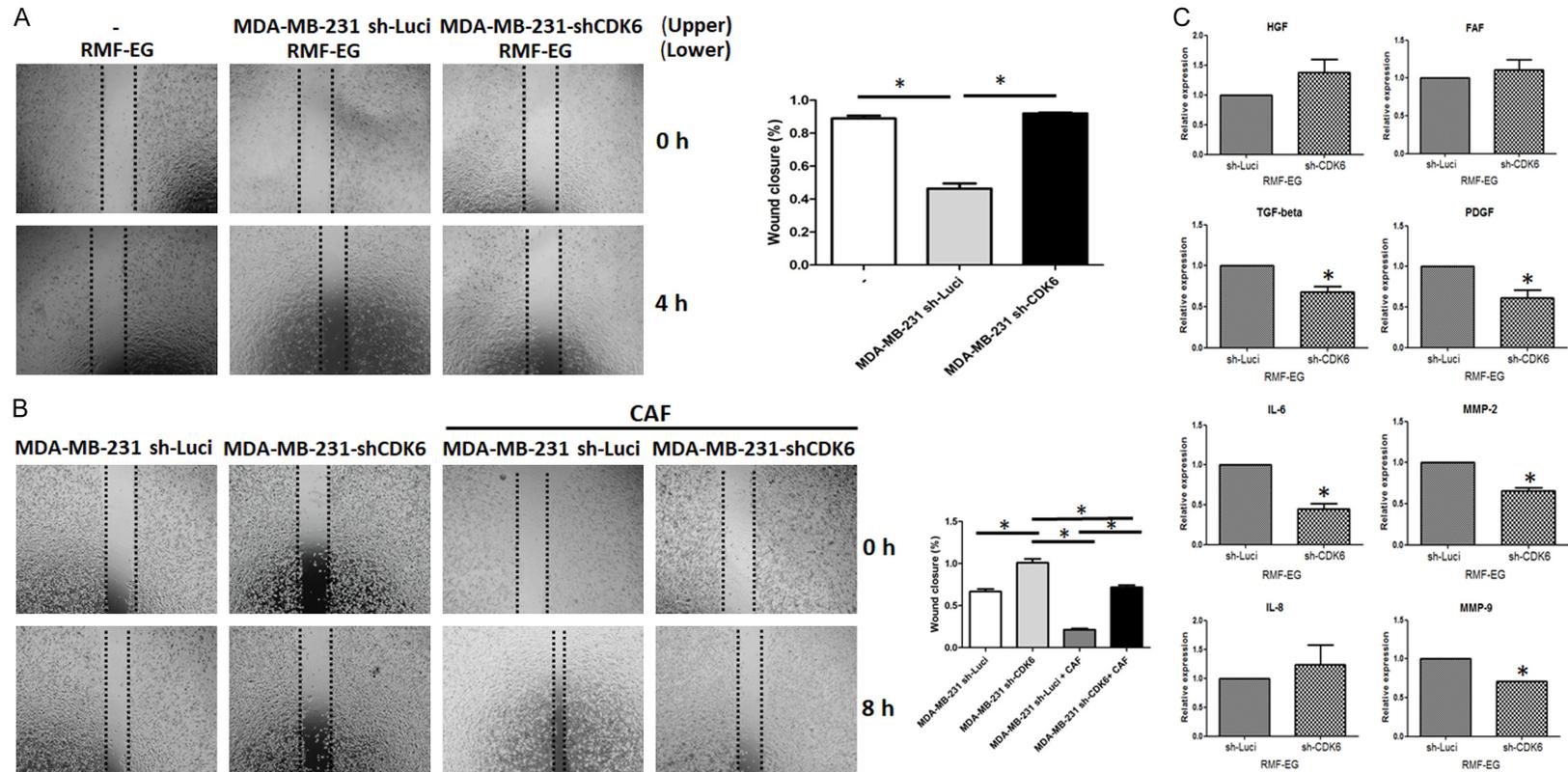
Figure 2. Knockdown of CDK6 impairs cancer-induced fibroblast activation and migration. A. For co-culture, 40,000 RMF-EG cells were seeded onto 24-well plates in 500 μL Dulbecco's modified Eagle's medium (DMEM). Additionally, 40,000 MDA-MB 231 or MDA-MB-231 sh-CDK6 pooling cells were seeded onto 0.2 μm Transwell inserts with 100 μL DMEM and co-cultured with RMF-EG cells for 48 h. Migration of RMF-EG cells into the scratch wound after 4 h was analyzed. Original magnification: 40 ×, scale bar: 500 μm. B. For the co-culture, 40,000 MDA-MB 231 or MDA-MB-231 sh-CDK6 pooling cells were seeded onto 24-well plates in 500 μL DMEM. Additionally, 40,000 cancer-associated fibroblasts were seeded onto 0.2 μm Transwell inserts in 100 μL DMEM and co-cultured with RMF-EG cells for 48 h. Migration of MDA-MB 231 and MDA-MB-231 sh-CDK6 pooling cells into the scratch wound after 8 h was analyzed. Original magnification: 40 ×, scale bar: 500 μm. C. The quantitative real-time polymerase chain reaction was used to investigate the mRNA expression of candidate genes in RMF-EG cells co-cultured with MDA-MB 231 or MDA-MB-231 sh-CDK6 pooling cells. Columns represent means of triplicate assays normalized to *GAPDH* expression (*$P < 0.05$).
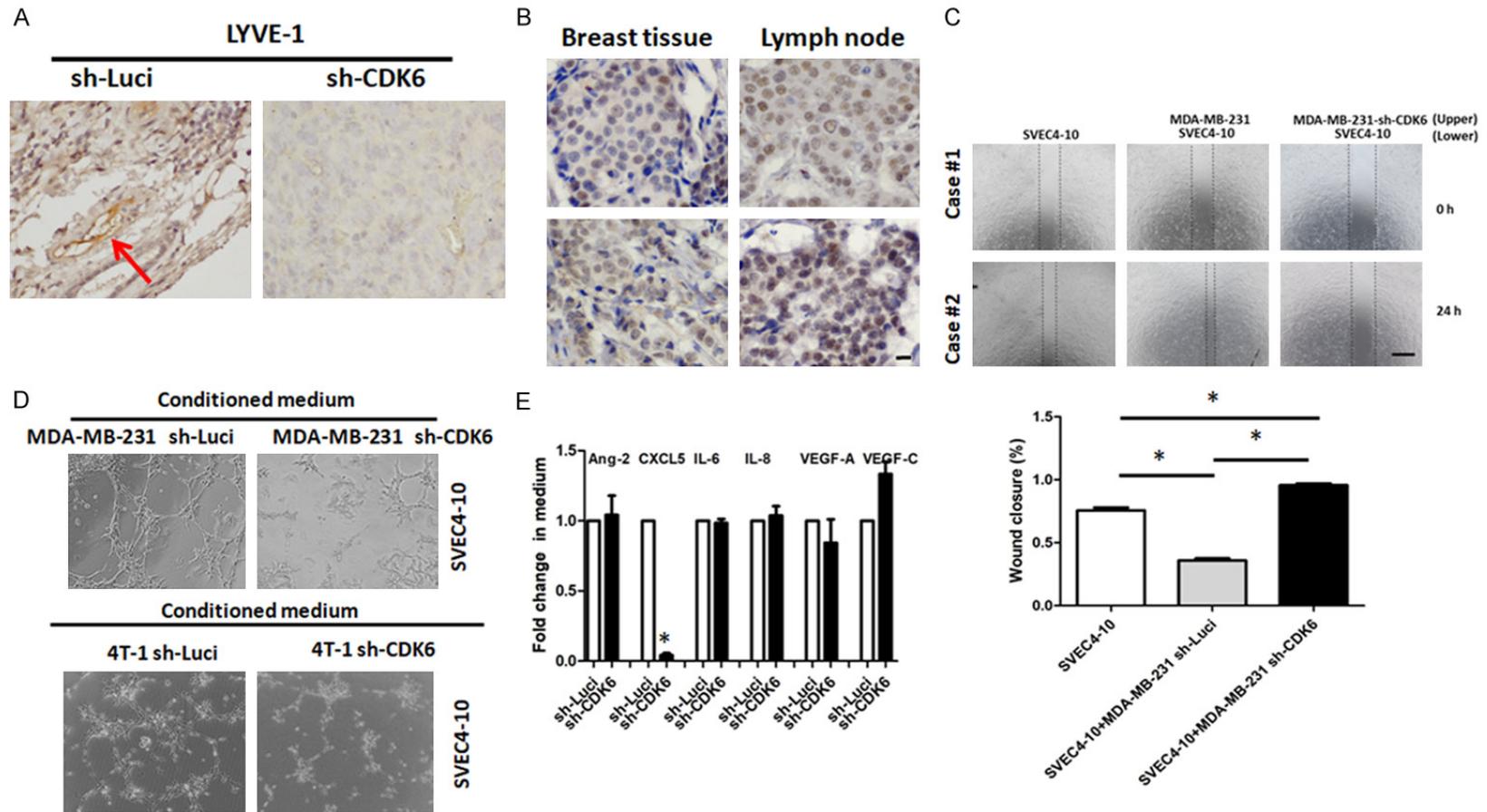
Figure 3. CDK6 in cancer cells mediates lymphangiogenesis. A. Levels of LYVE-1 in parental and sh-CDK6 transfected 4T1 cells, and representative images of breast cancer tissues with high or low expression of CDK6 in BALB/c mice. B. Representative immunohistochemical staining for CDK6 in primary tissue and lymph nodes of breast cancer patients. Original magnification: 40 ×, scale bar: 20 μm. C. For the co-culture, 40,000 SVEC4-10 cells were seeded onto 24-well plates in 500 μL Dulbecco's modified Eagle's medium (DMEM). Additionally, 40,000 MDA-MB 231 or MDA-MB-231 sh-CDK6 cells were seeded onto 0.2 μm Transwell inserts in 100 μL DMEM and co-cultured with SVEC4-10 cells for 48 h. Migration of SVEC4-10 cells into the scratch wound after 4 h was analyzed. Original magnification: 40 ×, scale bar: 500 μm. D. A total of 3 × 10^5 cells were seeded per 24 well with 1 mL of conditioned medium from either MDA-MB 231 or MDA-MB-231 sh-CDK6 pool or 4T1 or 4T1 sh-CDK6 pooling cells. Tube formation was assessed over the course of 4 h. E. Protein levels of candidate lymphangiogenesis-associated soluble factors were detected by enzyme-linked immunosorbent assay kits. Columns represent means of triplicate assays normalized to cell numbers (*$P < 0.05$).
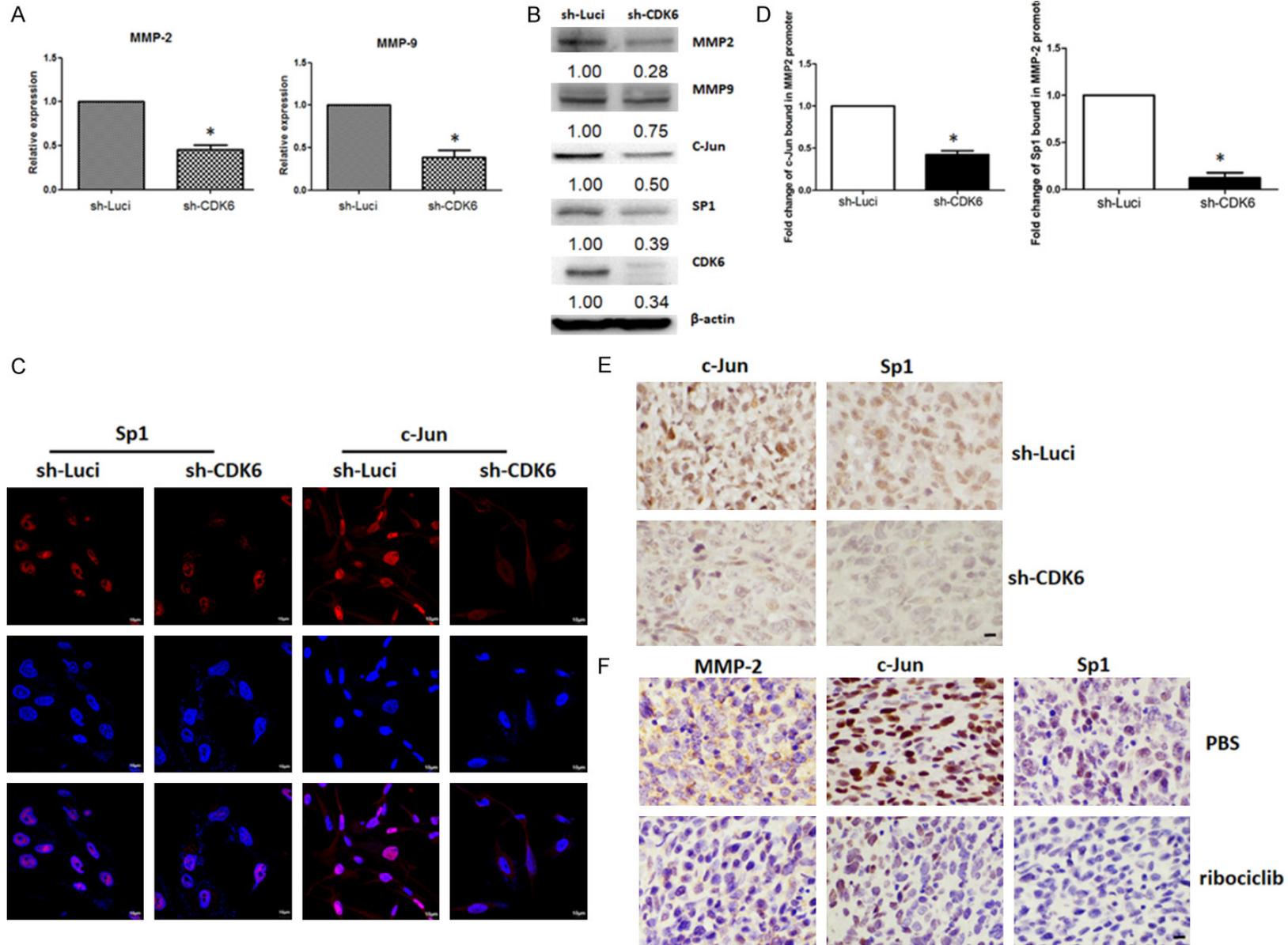
Figure 4. Knockdown of CDK6 suppresses the expression of *MMP2* through transcriptional regulation. A. The quantitative real-time polymerase chain reaction (qRT-PCR) was used to investigate the expression of *MMP2* and *MMP9* mRNAs in MDA-MB 231 and MDA-MB-231 sh-CDK6 pooling cells. Columns represent means of triplicate assays normalized to GAPDH (*$P < 0.05$). B. Protein expression levels of CDK6, c-Jun, Sp1, MMP-2, and MMP-9 in MDA-MB 231 and MDA-MB-231 sh-CDK6 pooling cells were determined by western blotting. C. Immunohistochemistry confirmed nuclear translocation of c-Jun and Sp1 in MDA-MB 231 and MDA-MB-231 sh-CDK6 pooling cells. D. A chromatin immunoprecipitation-qRT-PCR analysis was performed to determine the status of c-Jun and Sp1 in the MMP-2 gene promoter. Experiments were performed in triplicate (*$P < 0.05$). E. Representative immunohistochemical staining for c-Jun and Sp1, in tumors with high and low CDK6 expression. Original magnification: 40 ×, scale bar: 10 μm. F. Representative immunohistochemical staining for c-Jun and Sp1, in tumors with Ribociclib. Original magnification: 40 ×, scale bar: 10 μm.
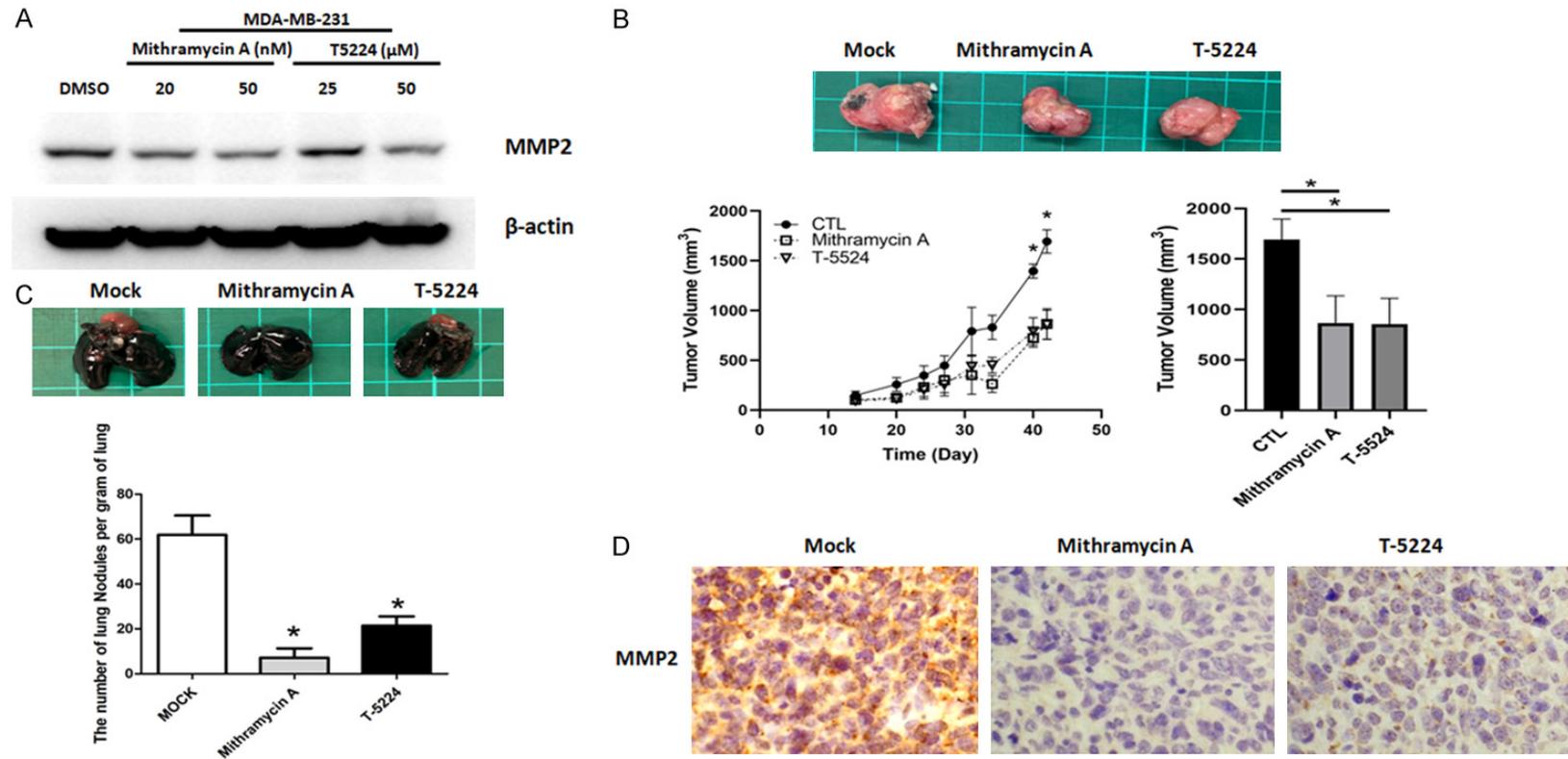
Figure 5. Treatment of *Mithramycin A* and T-5224 decreases MMP2 expression lung metastasis and tumor growth in vivo. A. Protein expression levels of MMP-2 were shown in MDA-MB 231 cells treated with *Mithramycin A* and T-5224 by western blotting. B. Representative images of breast cancer tissue with the treatment of *Mithramycin A* and T-5224 in BALB/c mice. Lower panel, quantitative analysis of tumor growth and size 45 days after tumor cell injection. Comparisons utilized the 2-tailed Student t test (*$P < 0.05$). C. Lungs were perfused with India ink after the mice were euthanized. Upper panel, representative images of lung nodules. Lower panel, numbers of visualized nodules per gram of lung tissue compared using the 2-tailed Student *t*-test (*$P < 0.05$). D. Representative immunohistochemical staining of MMP-2 in tumors treated with *Mithramycin A* and T-5224 in BALB/c mice.
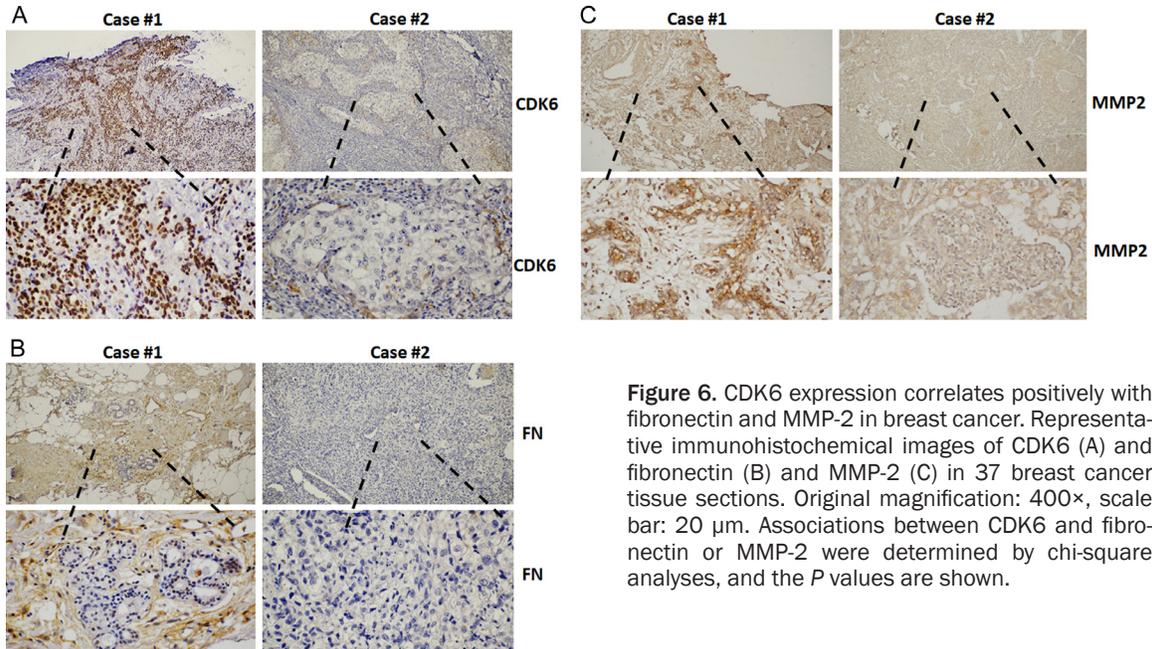
Figure 6. CDK6 expression correlates positively with fibronectin and MMP-2 in breast cancer. Representative immunohistochemical images of CDK6 (A) and fibronectin (B) and MMP-2 (C) in 37 breast cancer tissue sections. Original magnification: 400×, scale bar: 20 µm. Associations between CDK6 and fibronectin or MMP-2 were determined by chi-square analyses, and the *P* values are shown.

Table 1. Association of CDK6, Fibronectin expressions (ECM), and MMP-2 in TNBC tissues (n=37)

		Fibronectin (ECM) (%)		P-value	MMP-2 (%)		P-value
		Positive	negative		Positive	negative	
CDK6	High	11 (29.7)	1 (2.7)		10 (27.0)	2 (5.4)	
	Low	14 (37.8)	11 (29.7)	P < 0.05	9 (24.3)	16 (43.2)	P < 0.05

sue, tumor tissues from 37 TNBC patients were subjected to immunodetection of CDK6, as well as MMP-2 and fibronectin, to reveal the extents of angiogenesis and fibrosis, respectively. The positive correlation of CDK6 with both MMP-2 and fibronectin is consistent with a critical role for CDK6 in tumor progression (**Figure 6** and **Table 1**). This indicates that CDK6 may be a valuable therapeutic target in modulating the tumor microenvironment and reducing metastasis.

CDK6 inhibition combined with sorafenib treatment induces synthetic lethality of TNBC cells

To test the ability of CDK6 inhibition to induce cancer cell death, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was utilized. In addition, we applied the concept of synthetic lethality, which involves combining effects on two different genes to further decrease cancer cell viability beyond that caused by either treatment alone [28]. We performed high-throughput analyses using clinical and pre-clinical drugs to screen for agents that could synergistically combine with a CDK6 inhibitor. The toxicities of 31 different protein kinase inhibitors were measured on both MDA-MB-231 and sh-CDK6 MDA-MB-231 cells. Among the tested combinations, sorafenib, a RAF/MEK/ERK pathway inhibitor, had the most significant effect on inducing cell death in CDK6-knockdown cells (**Figure 7A**). Consistent with these findings, knockdown of CDK6 led to enhanced cytotoxicity upon sorafenib treatment exposure, as compared to that in parental MDA-MB-231 cells through the analysis of colonies (**Figure 7B**). As shown in **Figure 6C**, the amount of cells in the early and late apoptotic phase was increased in CDK6-depleted cells (sh-Luci: 9.15%; sh-CDK6: 47.17%). More importantly, the treatment of sorafenib elevated the apoptosis in CDK6-depleted cells. These data indicated that CDK6 knockdown further enhances the apoptotic effect of sorafenib in MDA-MB-231 cells (**Figure 7C**). Considering the results of previous results, the regulatory mechanism of CDK6 may be assumed to affect
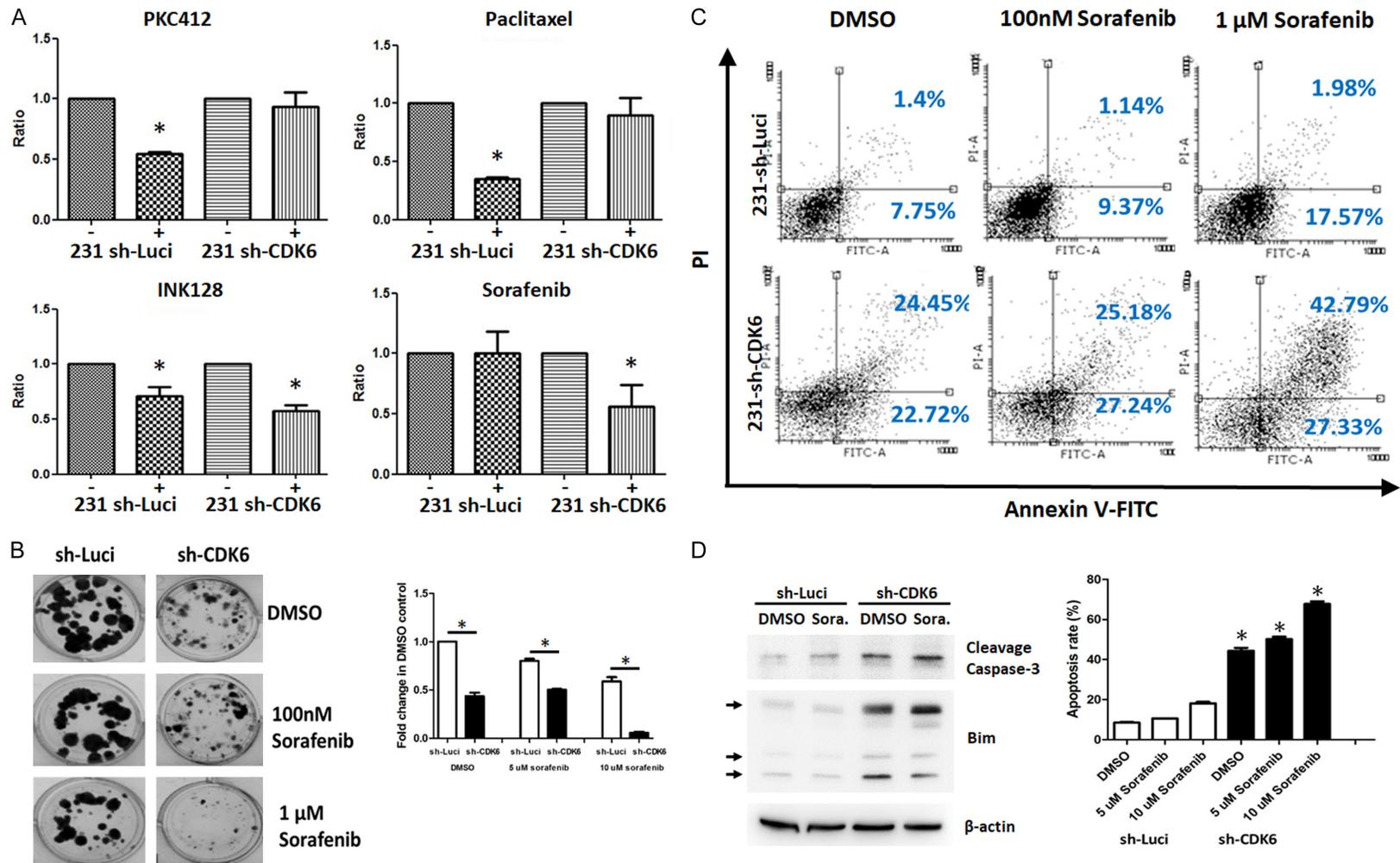
Figure 7. Knockdown of CDK6 enhances the cytotoxicity of sorafenib in breast cancer cells. A. MDA-MB 231 or MDA-MB-231 sh-CDK6 cells were treated with different compounds for 72 h. Cell viability was determined by the MTT assay (*P < 0.05). B. MDA-MB 231 or MDA-MB-231 sh-CDK6 cells were treated with sorafenib (0.1 or 1 µM) for 72 h, followed by culturing in fresh medium for 14 days. C. The percentage of apoptotic cells treated with indicated sorafenib for 72 h was detected via dual staining with annexin V-FITC and PI in MDA-MB 231 and MDA-MB-231 sh-CDK6 cells, followed by analysis of flow cytometry. The experiments were repeated three times. Between-group comparisons were performed using one-way ANOVA (*, P < 0.05). D. Protein expression levels of cleavage caspase-3, Bim and actin in MDA-MB 231 and MDA-MB-231 sh-CDK6 cells treated with vehicle and sorafenib for 72 h by western blotting.

the pro-survival proteins and pro-apoptotic proteins. Further, we found lower Bim and higher cleavage caspase-3 in CDK6-dpleted cells exposed with sorafenib treatment (**Figure 7D**). This result suggests that sorafenib could work with a CDK6 inhibitor to induce synthetic lethality in high CDK6 level/Rb-proficient TNBC cells.

Discussion

Uncontrolled cell cycle progression and proliferation are hallmarks of most types of cancer. Dysregulation of CDK6 expression and its activity are often associated with the development of cancers including lymphoma, leukemia, colorectal cancer, and estrogen receptor-positive breast cancer [29-31]. Notably, a previous report indicated that CDK6 was highly expressed in TNBC as compared to non-TNBC, and its overexpression correlated with a poor prognosis for TNBC [32]. However, the detailed mechanism still needed to be clarified. The present results agreed with those of previous studies showing that CDK6 expression correlated with an angiogenic and fibrotic tumor microenvironment. This suggests a positive correlation between CDK6 and a malignant tumor microenvironment.

The tumor microenvironment is an important regulator of cancer progression, a biomarker for prognosis, and a therapeutic target [33]. The microenvironment is composed of extracellular matrix, adipocytes, immune cells, endothelial cells, and fibroblasts [34]. Cancer cells interact with other cells within the microenvironment via soluble factors or intercellular receptor-ligand interactions. Different subtypes of breast cancer can create distinct microenvironments that favor malignant progression [35]. Among cells within the microenvironment, fibroblasts are a critical component for remodeling the environment to support cancer and participate in almost every step of cancer progression [36]. Fibroblasts interact with the tumor microenvironment through several factors that regulate inflammation and epithelial-to-mesenchymal events [33]. TGF-β, released by fibroblasts, affects TNBC tumor fibrosis, growth, and metastasis. These effects can be reversed by treatment with TGF-β antagonists and pirfenidone [37]. Fibroblast-regulated platelet-derived growth factor receptor (PDGFR)α, PDGFRβ, N2, and fibroblast activation protein α are highly expressed in TNBC and correlate with tumor invasiveness [38]. We found lower levels of IL-6, MMP-2, MMP-9, TGF-β, and PDGF in RMF-EG cells co-cultured with CDK6-deficient cells than in RMF-EG cells co-cultured with parental cells (**Figure 2C**). These results demonstrate that the level of CDK6 in cancer cells functions as a key modulator of fibroblast activation.

CDK6 has two major functions; first, its kinase-dependent function involves phosphorylating Rb and consequently releasing the transcription factor E2F. This promotes exit from the G1 phase, thereby increasing cell cycle progression [3-5]. Second, recent studies have also identified a novel kinase-independent function of CDK6 as a transcriptional regulator [39]. CDK6 potentially binds to transcription factors, such as NF-κB, STAT3, AP-1, SPI1, ETS, and PAX4, to function as a transcription cofactor [23, 39, 40]. CDK6 interacts with AP-1 and c-Jun to regulate the expression of *VEGFA* [39]. These findings indicate that CDK6 plays a dual role in carcinogenesis [40].

VEGF is increased aberrantly in TNBC tumors. It not only stimulates angiogenesis directly to provide vital nutrients for the tumor, but also boosts tumor-cell adhesion and migration via the cancer-endothelial cell interaction [41, 42]. Revealing the regulatory mechanism of the tumor-microenvironmental interaction that supports cancer progression will certainly help in the development of novel therapeutic strategies for TNBC. Herein, our findings identify CDK6 as a potential target for cancer therapy that inhibits recruitment of c-Jun and Sp1 to the MMP-2 promoter to block lung metastasis.

To date, three CDK4/6 inhibitors, palbociclib, ribociclib, and abemaciclib, have been used in clinical cancer treatment. They exert their anti-tumor effects by targeting the cell-cycle progression function of CDKs in Rb-proficient tumor cells [18]. Clinical trials with these inhibitors have been performed in several cancer types, including breast cancer, leukemia, and soft tissue sarcomas [18]. In addition to inducing cell-cycle exit, CDK4/6 inhibitors may have other anticancer effects. For example, CDK4/6 inhibition promotes glycolytic and oxidative metabolism, increasing the number of mitochondria and reactive oxygen species accumulation via an Rb-dependent mechanism [43]. CDK4/6 inhibitor treatment also has been pro-

posed to increase PD-L1 expression by blocking its degradation, as well as by increasing the number of PD-L1-expressing T cells and their infiltration [44, 45]. Notably, CDK4-deficient supporting cells, but not cancer itself, also diminish the aggressive progression of oligodendroglioma [46]. Our data suggest a role for CDKs in developing the tumor microenvironment. Our findings also show that CDK6 plays an important role in the interaction between tumor and supporting cells, suggesting that inhibition of CDK6 may target the tumor microenvironment, resulting in a therapeutic effect.

An increasing number of early-phase clinical trials have examined the therapeutic effects of drug combinations [18]. According to the synthetic lethality theory, such combined treatments can minimize drug resistance and maximize antitumor effects [28]. In some types of cancer, resistance can be overcome by including a CDK4/6 inhibitor with signaling pathway-targeted inhibitor treatments, such as MEK and phosphoinositide 3-kinase/Akt inhibitors [47, 48]. However, some have also reported an antagonistic effect of combined treatments used simultaneously due to interference between the treatments [48]. These findings demonstrate the necessity of designing an appropriate drug delivery schedule that depends on cancer type and biomarkers. After screening currently available signaling pathway-targeted drugs, we found that combined inhibition of CDK6 and Raf achieved the best cytotoxic effect in TNBC cells. Our results suggest that CDK6 inhibition could be a personalized treatment that works synergistically with chemotherapy drugs to improve overall therapeutic efficacy. However, further studies need to be conducted to determine the optimal sequence, timing, and dosages of combined CDK6/Raf inhibitor treatments for improving the therapeutic effect.

Conclusion

Summary, we provide evidence that CDK6 plays a significant role in the interaction between cancer and its microenvironment. CDK6 levels correlated positively with an increased angiogenic and fibrotic microenvironment in TNBC cells. This suggests that CDK6 can serve as both a biomarker and therapeutic target. CDK6 was involved in the cancer-microenvironmental interaction via the regulation of secreted factors that activated endothelial cells and fibroblasts. We also demonstrated that the Raf inhibitor, sorafenib, exerted a synergistic effect with CDK6 inhibition to induce synthetic lethality in TNBC cells. Overall, these results indicate that combining CDK6 inhibition with a Raf inhibitor could be a personalized treatment for the Rb-positive subpopulation of patients with TNBC.

Disclosure of conflict of interest

None.

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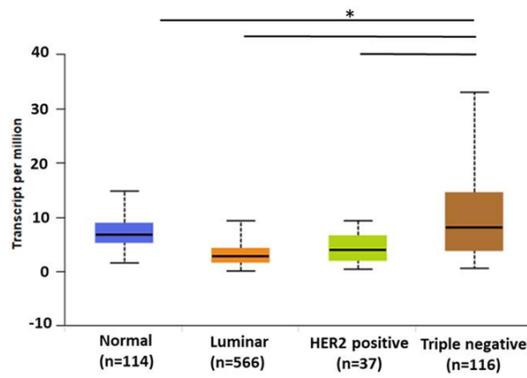
Figure S1. The CDK6 expression was further evaluated by the UALCAN database according to different subtypes. The number in parentheses indicates sample size in each group. *P < 0.05, as compared between each group.
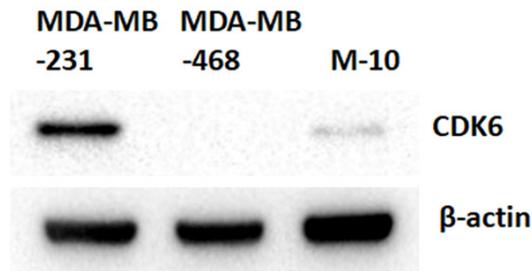
Figure S2. Expression of CDK6 in normal human mammary epithelial cell (M10) and two TNBC cell lines (MDA-MB-231 and MDA-MB-468). Cell lysates were immunoblotted by anti-CDK6. Actin was used as a loading control. Experiments were performed independently at least three times.
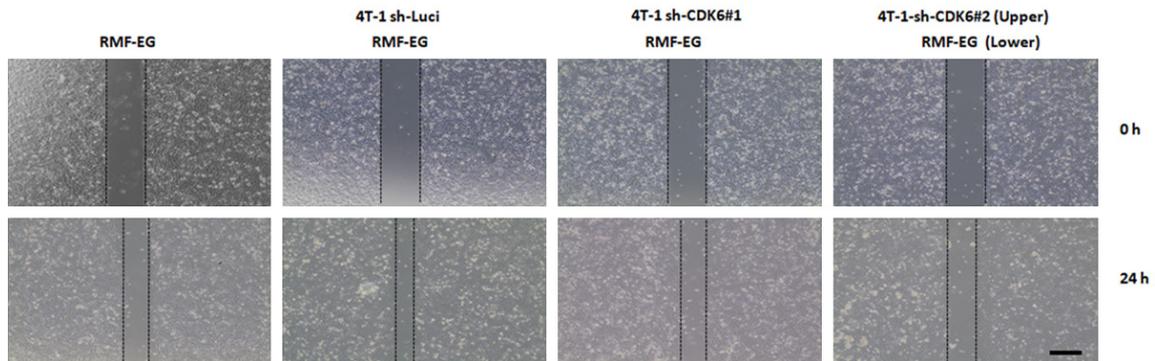
Figure S3. The migration of fibroblast was performed in co-culture with 4T1 and CDK6-deficient 4T1 cells. 40,000 RMF-EG cells were seeded onto 24-well plates in 500 μL Dulbecco's modified Eagle's medium (DMEM). Additionally, 40,000 4T1 or 4T1 sh-CDK6 pooling cells were seeded onto 0.2 μm Transwell inserts with 100 μL DMEM and co-cultured with RMF-EG cells for 48 h. Migration of RMF-EG cells into the scratch wound after 24 h was analyzed. Original magnification: 40 ×, scale bar: 500 μm.

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Figure S4. The migration of 4T1 was performed in co-culture with CAF cells. 40,000 4T1 or 4T1 sh-CDK6 pooling cells were seeded onto 24-well plates in 500 μL DMEM. Additionally, 40,000 cancer-associated fibroblasts were seeded onto 0.2 μm Transwell inserts in 100 μL DMEM and co-cultured with RMF-EG cells for 48 h. Migration of 4T1 or 4T1 sh-CDK6 pooling cells into the scratch wound after 8 h and 24 h was analyzed. Original magnification: 40 ×, scale bar: 500 μm.
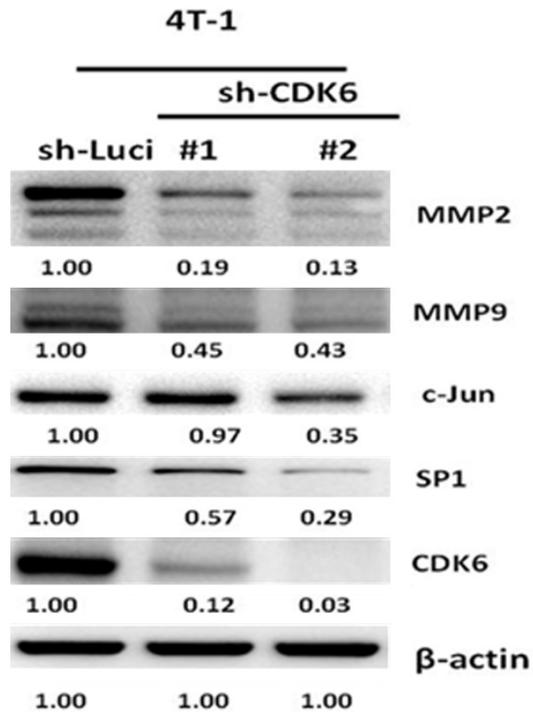
Figure S5. Protein expression levels of CDK6, c-Jun, Sp1, MMP-2, and MMP-9 in 4T1 and 4T1 sh-CDK6 pooling cells were determined by western blotting.

Figure S6. Immunohistochemistry confirmed nuclear translocation of c-Jun and Sp1 in 4T1 and 4T1 sh-CDK6 pooling cells.